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Ecology and Epidemiology

Susceptibility of the Banana Inflorescence to Blood Disease

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ABSTRACT

The bacterium *Ralstonia syzygii* subsp. *celebesensis* causes Blood disease of banana, a vascular wilt of economic significance in Indonesia and Malaysia. Blood disease has expanded its geographic range in the last 20 years and is an emerging threat to Southeast Asian banana production. Many aspects of the disease cycle and biology are not well understood, including the ability of different parts of the female and male inflorescence of banana to act as infection courts. This study confirms that the banana varieties of Cavendish, and Kepok 'Kuning' are susceptible to Blood disease and that an inoculum concentration of 10^2 CFU/ml of *R. syzygii* subsp. *celebesensis* is adequate to initiate disease after pseudostem inoculation. Data show that infection occurs through both the male and female parts of a banana inflorescence and the rachis when snapped to remove the male bell. The infection courts are the female flowers, the male bell bract scar, the male bell flower cushion, the snapped rachis, and deflowered fingers. The location of these infection courts concurs with the dye studies demonstrating that dye externally applied to these plants parts enters the plant vascular system. Thus, the hypothesis is supported that infection of *R. syzygii* subsp. *celebesensis* occurs through open xylem vessels of the male and female parts of the banana inflorescence.

Keywords: bacterial pathogens, epidemiology, etiology

Bananas (*Musa* sp.) are an important global commodity with an annual production of 162 million tonnes (Mt) in 2016, of which most is sold and consumed in the country of production, whereas 15% is exported to international markets (FAOSTAT 2018). Southeast Asia produced 17 Mt of dessert bananas in 2016, most of which was grown in Indonesia (7 Mt) and the Philippines (5.8 Mt), with lower levels of production in Vietnam (2 Mt), Thailand (1 Mt), Laos (0.8 Mt), and Malaysia (0.3 Mt) (FAOSTAT 2018). In Southeast Asia, bananas are especially important for smallholder growers providing a source of food and income throughout the year (FAOSTAT 2018; Robinson 1996). The cooking bananas in the Kepok (ABB) group are the main banana varieties grown and traded in Indonesia by smallholder growers, with the Kepok 'Kuning' (syn: Nipah) variety popular for its growth and consumption characteristics.

Bananas are affected by several bacterial wilt diseases, of which Blood disease caused by *Ralstonia syzygii* subsp. *celebesensis* is the only one occurring in Indonesia (Ray et al. 2021; Safni et al. 2014). The symptoms of Blood disease are systemic and expressed as rotting of the fruit pulp, wilting and necrosis of the leaves, uneven bunch ripening, necrosis of the male bell, and vascular staining of the fruit pedicels, rachis, peduncle, and pseudostem (Fig. 1). Blood disease causes significant crop losses in Indonesia and Malaysia, where it mainly affects the popular, but highly susceptible, Kepok banana varieties (ABB) (Eden-Green and Sastraatmadja 1990; Gäumann 1921; Geddes 1992; Teng et al. 2016). The recent rapid geographic expansion of the pathogen in Indonesia and more recently to Malaysia may have severe implications for banana production in Southeast Asia and beyond (Ray et al. 2021; Safni et al. 2014; Teng et al. 2016).

The recent geographic expansion of Blood disease raises questions concerning the bacteria's ability to spread and disseminate. Although it is tempting to look at the epidemiology of other bacterial wilts in banana, it is important to realize that Blood disease differs from Moko, caused by *Ralstonia solanacearum sensu stricto*, originating from the Americas, in that it lacks flagella (Eden-Green and Sastraatmadja 1990; Roberts et al. 1990; Safni et al. 2014). The flagellated *R. solanacearum* move toward plant host roots using chemotaxis and flagellar motility, which are important epidemiological factors (Clough et al. 1997; Tans-Kersten et al. 2001; Yao and Allen 2006). The lack of flagella likely hampers movement in the soil of *R. syzygii* subsp. *celebesensis* compared with *R. solanacearum* and warrants specific investigations into the epidemiology and disease cycle of Blood disease.

Local transmission of *R. syzygii* subsp. *celebesensis* has been hypothesized to be predominantly via insects that visit the male bell and mechanically transfer the bacterium from diseased to healthy banana plants (Buddenhagen 2009; Stover and Espinoza 1992). Long-distance spread is thought to occur through the movement of infected planting material (Gäumann 1923; Ray et al. 2021). Little to no evidence supports claims that transmission also occurs through contaminated tools, soil, water, nematodes, bats, birds, and infected fruit sent to market (Gäumann 1921, 1923; Safni et al. 2018; Subandiyah et al. 2005). Resistance to *R. syzygii* subsp. *celebesensis* is not known to occur within *Musa* spp. (Gäumann 1921), but banana varieties with retained bracts or without a male bell, such as Kepok 'Tanjung', do not generally become diseased in the field (Buddenhagen 2009; Hermanto and Emilda 2013). The overall lack of known resistance to Blood disease emphasizes the need for alternative disease management options.

Buddenhagen (2009) hypothesized that *R. syzygii* subsp. *celebesensis* predominantly infects the banana plant through xylem vessels that remain open at the male bell flower cushion after flowers abscise, although this claim was not supported by evidence. *R. solanacearum*, a related bacterium, thrives in plant xylem vessels, although infection predominantly occurs through the roots of susceptible hosts (Lowe-Power et al. 2018). These bacteria are adapted to

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and exploit the flowing nutrient-poor xylem fluids through a quorum-sensing system to modulate between dispersal and adhering to the xylem wall by forming biofilms (Lowe-Power et al. 2018; Tran et al. 2016). *R. solanacearum* becomes systemic, primarily colonizing plant xylem, but can degrade vessel walls and colonize adjacent cells and vessels (Digonnet et al. 2012; Kelman 1953; Lowe-Power et al. 2018).

Dye uptake studies are commonly used to elucidate xylem function and flow. Water-soluble apoplastic dyes were particularly informative in establishing the role of xylem in the postveraison grape berry (Bondada et al. 2005; During et al. 1987). The pattern of tissue staining resulting from the external application of dye to the cut berry pedicel is presumed to replicate fluid flowing through the xylem because of naturally occurring hydrostatic (tension) gradients (Bondada et al. 2005; During et al. 1987).

One way to control Blood disease is to reduce the risk of infection. Because infection is hypothesized to occur mainly through the inflorescence, it is important to understand the floral biology of banana. The banana plant produces a terminal inflorescence that emerges from the center at the top of the pseudostem (Robinson 1996). After emergence, large bracts initially cover each cluster of banana flowers within the inflorescence; usually each day, one to three bracts lift to reveal the maturing flowers (Fig. 2A) (Stover and Simmonds 1987; Robinson 1996). The open bracts are retained in some varieties whereas in others they abscise and fall to the ground, leaving potentially exposed xylem vessels (Fig. 2B) (Buddenhagen 2009; Stover and Simmonds 1987).

The banana inflorescence has three types of flowers; the first to emerge are the female flowers, followed by neuter or hermaphrodite flowers, and then the male bell containing male flowers (Stover and Simmonds 1987). The female flower has an elongated inferior ovary approximately two-thirds of the length of the whole flower (Karamura et al. 2011) (Fig. 2A). This elongated ovary eventually develops into the banana fruit (finger), which in parthenocarpic plants contains no seed and becomes edible (Stover and Simmonds 1987). The male flowers are revealed after the female flowers and are attached to cushions on the peduncle (Karamura et al. 2011; Stover and Simmonds 1987). Once revealed, the male flowers readily abscise, revealing the flower cushion (Buddenhagen 2009; Karamura et al. 2011; Stover and Simmonds 1987) (Fig. 2B).

To elucidate the infection biology and epidemiology of Blood disease, the overall objective of this study was to investigate different parts of the banana inflorescence for their ability to act as infection courts. Therefore, we conducted several screenhouse and field trials to address the following questions: (i) Is there a relationship between inoculum concentration and disease development? (ii) Can the rachis (when snapped during denaveling), male bell bract scars, male bell flower cushions, female flowers, deflowered fingers, and maturing bunch act as infection courts? (iii) Are the xylem vessels of the banana inflorescence open and exerting capillary action? (iv) Can *R. syzygii* subsp. *celebesensis* infect via open xylem vessels? Addressing these questions is critical to improving our knowledge of the epidemiology of Blood disease, a prerequisite to developing effective disease management strategies.

MATERIALS AND METHODS

Bacterial isolate and preparation of plant inoculum. A locally sourced isolate of *R. syzygii* subsp. *celebesensis* was a requirement for conducting research at our field site in Bantul, Special Region of Yogyakarta, Indonesia, to prevent introducing a new strain to the area. For this reason, isolate JR3824 obtained from a symptomatic banana plant originating from Bantul was used for all subsequent experimentation (Ray et al. 2021). Isolate JR3824, previously identified as *R. syzygii* subsp. *celebesensis* and confirmed pathogenic (Ray et al. 2021), was retrieved from cultures stored in microtubes

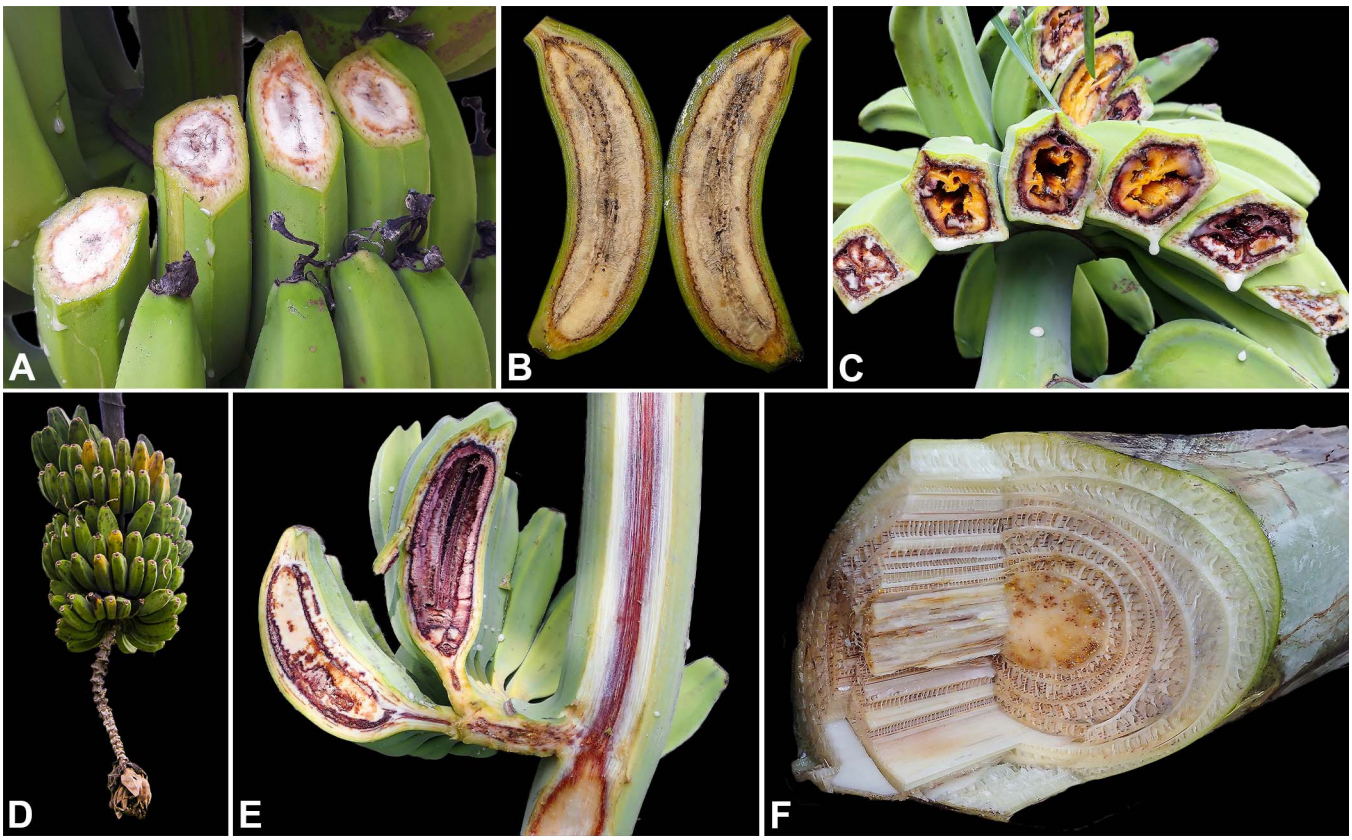

Fig. 1. Symptoms of Blood disease in bananas caused by *Ralstonia syzygii* subsp. *celebesensis.* Internal pulp rot and discoloration of green fingers; **A and B,** Cavendish and **C,** Kepok 'Kuning'. **D,** Necrosis of male bell and uneven ripening of Kepok 'Kuning' fingers. Characteristic vascular staining in Kepok; **E,** rachis, pedicel, fruit, and **F,** pseudostem.

containing sterile water at room temperature in the dark. Cultures were revived and inoculum was prepared as described previously (Ray et al. 2021), and then they were adjusted to the required concentration as detailed below using a spectrometer (OD600 Implen, Munich, Germany), followed by a serial dilution to confirm viability and concentration. To prevent cross-contamination during plant maintenance, experimental set-up, and monitoring activities, sanitation of tools and hands was routinely carried out using 80% ethanol.

Plants. Commercially produced banana plants (*Musa* spp.) originating from tissue culture were used for the potted and field experiments conducted in Indonesia. Plants were sourced from Institute Plants Center, Magelang, Central Java, Indonesia.

Potted plants. The banana variety Kepok Kuning (Kepok) was grown in potting mix in 30-cm pots. Plants were transferred to a shade house and grown in natural light under a maximum day temperature of 38°C and a minimum night temperature of 23°C.

Field-grown plants for inflorescence inoculation experiments. Kepok and Cavendish bananas were planted at a field trial site in Bantul, Special Region of Yogyakarta, Java, Indonesia. Plants at the five- to six-leaf stage were planted in October 2018 into a duplicated block design that included 190 Cavendish and 170 Kepok plants. To replicate commercial production, plants were de-suckered and de-leafed as required. The plant inflorescence was bagged soon after emergence to prevent natural infection with Blood disease using ultraviolet light stabilized cloth banana bunch sleeves (bags) (SL04-C900 Crownpack, Kunda Park, QLD, Australia). The bags were secured to the peduncle using a cable tie and closed below the inflorescence to prevent insect entry (Fig. 2C). The abscised bracts and flowers were removed from the bottom of the bags every 7 to 10 days. When the bell outgrew the initial bag, a second bag was attached over the first and sealed at the rachis using a zip tie to maintain complete protection.

Field-grown plants for assessment of xylem status experiments. More than 200 plants of the Cavendish banana variety originating from tissue culture (Maroochy Research Facility, Queensland Department of Agriculture and Fisheries, Nambour, Queensland, Australia) were planted at Coastal Plains Research Farm, Northern Territory, Australia, in February 2019. The plants were de-suckered as required.

Bacterial isolation and confirmatory PCR. To confirm that symptoms observed during all banana inoculation experiments were those of Blood disease caused by *R. syzygii* subsp. *celebesensis,* isolations were carried out as described previously (Ray et al. 2021). Bacteria that are characteristic of *R. syzygii* subsp. *celebesensis* were retrieved from each symptomatic treatment for all varieties on each repeat of the experiment. DNA was extracted from the isolates and used to confirm that *R. syzygii* subsp. *celebesensis* was the causal agent using a specific diagnostic assay involving the use of primers (121F/121R), as described previously (Rincón-Flórez et al. 2021).

The effect of inoculum concentration. *Inoculum concentration and disease development.* To determine the level of inoculum needed for infection and subsequent disease expression, potted Kepok plants grown as described above at the nine-leaf growth stage were treated with inoculum at different concentrations. The inoculum was injected into the pseudostem approximately 3 cm above the soil line using a 25-G needle. The inoculum of isolate JR3824 of *R. syzygii* subsp. *celebesensis* was prepared as described above and adjusted to approximately 10^{10} CFU/ml and then serially diluted 10-fold to 10^{2} CFU/ml, and water was used as a negative control. Each treatment included three plants.

Symptoms were assessed 10 to 18 days after inoculation. Plants expressing yellowing, necrosis, and wilting of leaves were considered infected with *R. syzygii* subsp. *celebesensis*. For confirmation, one replicate from each treatment was harvested and assessed for internal vascular staining. In addition, isolations were carried out on three arbitrarily chosen symptomatic plants, one from each of the concentrations 10^{8}, 10^{6}, and 10^{5} CFU/ml, and the causal agent was confirmed using PCR as described above.

Inflorescence infection courts. The following experiments were conducted to evaluate which parts of the banana inflorescence can act as infection courts. Different plant parts of field-grown Kepok and Cavendish plants were inoculated with 10^{7} CFU/ml of isolate JR3824 and evaluated for disease development. Plants were arbitrarily chosen at the growth stage required for inoculation of the specific inflorescence parts. The treatments assessed were inoculation of (i) freshly snapped rachis during denaveling; (ii) the male bell; (iii) newly exposed male bell bract scars from naturally abscised bracts; (iv) newly exposed male flower cushions from naturally abscised flowers; (v) newly emerged open female flowers, which included the stigma, style, stamens, ovary, perianth, with bracts still attached; (vi) fingers freshly deflowered; and (vii) maturing bunch, denaveled >1 week previously.

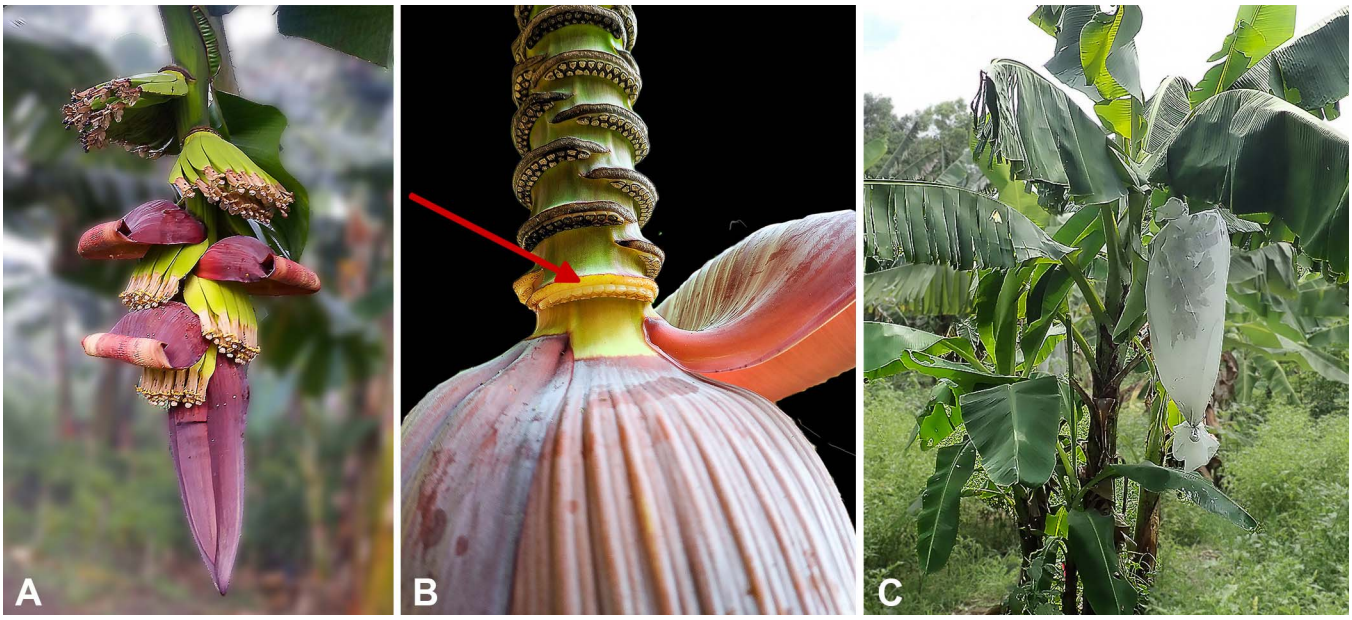

Fig. 2. Field-grown bananas. **A,** Cavendish inflorescence showing bracts lifting to reveal female flowers. **B,** Kepok 'Kuning' male bell. Arrow indicates recently revealed bract scar and a row of flower cushions below. **C,** Cavendish inflorescence protected at emergence with sealed cloth bag.

The female flowers were spray-inoculated with 750 µl of inoculum, and great care was taken to prevent overspray from reaching bract attachment sites. For each male bell, two to three male bell bract scars or two to three flower cushions were inoculated with 100 µl of inoculum using a pipette. Where required, either the bract scar or the flower cushion was protected using petroleum jelly (Vaseline) as a barrier to prevent cross-contamination during these inoculations. The snapped rachis, the male bell, deflowered fingers, and fingers of a maturing bunch were locally spray-inoculated with 1.5 ml of the inoculum. All inoculations took place from 9 a.m. to 12 a.m. After inoculation, the cloth bags were resealed to prevent infection from other sources. Each treatment included at least three plants for Cavendish and Kepok, except the deflowering treatment, which was only applied to Cavendish because Kepok female flowers naturally abscise. Controls consisted of at least three Cavendish and Kepok plants bagged to prevent infection. The experiment was fully repeated once at a later date.

Plants were assessed for expression of symptoms after 5 and 9 weeks. For each time point, at least three banana fingers were cut from at least three different hands using a knife, and plants were rated as positive when symptoms of pulp rot and discoloration characteristic of Blood disease were observed. Plants were rated as negative when no symptoms were visible after 9 weeks, including a check for internal vascular staining in the bunch peduncle and the pseudostem. Tissues with vascular staining symptoms were collected and subjected to bacterial isolation to confirm the presence or absence of *R. syzygii* subsp. *celebesensis.* Isolations were carried out from symptomatic treatments to confirm the causal agent using PCR as described above. Differences between inoculation treatments at 9 weeks were performed for each banana variety using one-way analysis of variance coupled with Tukey's post hoc test ($P \leq 0.05$) using SPSS version 27 ($P \leq 0.05$). To assess differences of infection after 9 weeks between Kepok and Cavendish varieties, a *t* test ($P \leq 0.05$) using SPSS version 27 was performed.

Inflorescence xylem vessel status and capillary action. To determine whether infection can occur through exposed xylem vessels, we sought to address the question if the xylem vessels of the banana inflorescence are open and subject to capillary action. Horticultural blue dye (Yates, Victoria, Australia) was applied to various inflorescence plant parts and evaluated for entry and movement within the plant. Cavendish plants were arbitrarily selected at the correct growth stage. Dye was applied using a fine sable artist's brush (#2, X72 Finest Kolinsky, Victoria, Australia) to various freshly revealed or newly emerged parts of banana inflorescences, except the flower treatment, which was spray-inoculated. All inoculations took place from 8 a.m. to 10 a.m., and all treatments were assessed after approximately 24 h.

The first experiment assessed xylem status and capillary action using dye inoculation at (i) female bract scar, (ii) rachis snapped during denaveling, (iii) male flower cushion, and (iv) male bract scar. Treatments were visually assessed for presence of blue dye inside the plant through dissection and measuring the distance that the dye moved through the xylem into the rachis and pseudostem in centimeters. Each treatment included three plants. Controls included two banana plants with freshly revealed female flowers and two plants with a male bell.

The second experiment assessed the potential for entry into the xylem of the banana finger through dye inoculation at (i) flowers, (ii) compound tepal tip, (iii) deflowered fingers, and (iv) stigma. Twelve fingers on each of the three most recently revealed hands were treated and assessed. The treatments of flowers, stigma, and compound tepal tip used freshly revealed female flowers. Deflowering was conducted after the male bell had formed, replicating commercial practice. Controls included two banana plants with freshly revealed female flowers and two with a male bell and deflowered fingers. After 24 h, the plants of all treatments were harvested and transported directly to the lab for assessment, except for the treatment flowers, in which the excess dye caused by spray inoculation was washed off with water after harvest before assessment. Treatments were visually assessed after dissection of the plant part and rated as positive when the blue dye was clearly seen inside the banana finger's vascular tissue. Each treatment included three plants.

RESULTS

The effect of inoculum concentration. Potted banana plants inoculated by stem injection with 10^{10} to 10^4 CFU/ml *R. syzygii* subsp. *celebesensis* started expressing visible leaf wilt and chlorosis 12 days after inoculation (Table 1). Plants inoculated with 10^3 and 10^2 CFU/ml first expressed visible symptoms 14 days after inoculation. All Kepok plants inoculated with 10^{10} to 10^2 CFU/ml of *R. syzygii* subsp. *celebesensis* expressed visible symptoms of Blood disease after 18 days and later died, while all the control plants remained healthy (Table 1; Fig. 3). The pattern of symptom progression over time was identical for all inoculated potted plants. The leaves lost turgor and began to wilt, becoming progressively more chlorotic and wilted over time, and as the disease progressed, the leaves folded at the petiole and became necrotic (Fig. 3). Internally, the vascular strands in the pseudostem stained red-brown and the corm discolored (Fig. 3F). Bacteria reisolated from symptomatic tissues were confirmed to be *R. syzygii* subsp. *celebesensis* based on PCR diagnostics. The median inoculum concentration of those that expressed symptoms at 12 days was 10^7 CFU/ml, which was then used for inoculations in subsequent experiments in this study.

Inflorescence infection courts. Symptoms of Blood disease observed after inoculation of the banana inflorescence included rot and discoloration of the fruit pulp (Fig. 1A to C) and vascular staining in the bunch peduncle and the pseudostem (Fig. 1E and F). Banana inflorescence inoculations of the rachis when snapped during denaveling, male bell, male bell flower cushion, male bell bract scar, and female flowers resulted in at least one plant from each of these treatments developing symptoms of Blood disease for both Cavendish and Kepok varieties after 9 weeks (Table 2). Inoculation of deflowered Cavendish fingers also resulted in one out of eight plants developing symptoms of Blood disease. Inoculation of the denaveled maturing bunch and the control plants remained asymptomatic (Table 2). The identity of bacteria reisolated from symptomatic tissues was confirmed by DNA extraction and PCR diagnostics as *R. syzygii* subsp. *celebesensis.*

Inoculation of the snapped rachis, the male bell, and the male bell flower cushion for Kepok and the snapped rachis, the male bell, and the male bell bract scar for Cavendish resulted in infection; subsequent symptom development was significantly different from the control at $P \leq 0.05$ after 9 weeks (Table 2). In contrast, a significantly ($P \leq 0.05$) lower disease incidence was observed for inoculation of female flowers for both varieties (Table 2).

There was no change in the number of plants expressing Blood disease symptoms from 5 to 9 weeks postinoculation for all treatments, except for the female flowers of Kepok and Cavendish plants. After inoculation of the inflorescence, one Kepok plant

TABLE 1. Relationship between inoculum concentration and symptom development for Blood disease assessed by stem injection of potted Kepok 'Kuning' banana plants with *Ralstonia syzygii* subsp. *celebesensis*, 10 to 18 days postinoculation

	Concentration of *Ralstonia syzygii* subsp. *celebesensis* in CFU/ml[z]									
Days	10^{10}	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	Control
10	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
12	2/3	2/3	2/3	1/3	1/3	1/3	2/3	0/3	0/3	0/3
14	2/3	3/3	3/3	2/3	2/3	2/3	3/3	2/3	1/3	0/3
16	3/3	3/3	3/3	3/3	2/3	3/3	3/3	2/3	2/3	0/3
18	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3

[z] Number of symptomatic plants/total number of inoculated plants.

developed symptoms after 5 weeks, and two had developed symptoms after 9 weeks. Cavendish plants expressed no symptoms at 5 weeks, and one had developed symptoms after 9 weeks. There was no significant difference in the infection of the varieties Kepok and Cavendish ($P \geq 0.05$) 9 weeks after inoculation.

Inflorescence xylem vessel status and capillary action. Blue dye, when applied externally to the female bract scars, the snapped rachis, male flower cushion, and male bract scar, was observed to have entered and stained the xylem inside the plant connected with that plant part after 24 h (Table 3; Fig. 4A to C). There was no evidence of blue discoloration in the dissected male and female control plants. Dye applied externally to a specific plant part of the inflorescence was observed to enter the xylem and continue into the plant, staining the rachis vascular tissue and sometimes the true stem (Table 3; Fig. 4). The average distance the dye traveled into the plant through the female bract scar was 167 cm, for the snapped rachis it was 92 cm, for the male flower cushion it was 47 cm, and for the male bract scar it was 23 cm (Table 3).

Blue dye, when sprayed on the female flowers of a banana inflorescence and painted on the compound tepal tip of female flowers and on deflowered fingers, was observed to enter the xylem of the three most recently revealed hands after 24 h (Figs. 4D and E and 5). In contrast, blue dye painted on the female flower stigma was not observed to enter through this plant part into the style tube or the finger's vascular tissue (Figs. 4F and 5). Dye painted on the tepal tip and deflowered fingers of the most recently revealed hand (hand one) was relatively more receptive to dye penetration than the earlier revealed hand (hand three) (Fig. 5). There was no evidence of blue discoloration in the dissected female flowers and fingers used as controls.

TABLE 2. Symptom expression of banana Blood disease in field-grown Kepok 'Kuning' and Cavendish banana varieties 9 weeks postinoculation of the male and female parts of the banana inflorescence, snapped rachis, deflowered maturing fingers, and maturing fingers

Treatment	Kepok[z]	Cavendish[z]
Snapped rachis	6/6 b	8/8 c
Bell	6/6 b	8/8 c
Male bell flower cushion	6/6 b	3/8 ab
Male bell bract scar	1/6 a	6/8 bc
Female flowers	2/6 a	1/8 a
Deflowered fingers	N/A	1/8 a
Maturing fingers	0/6 a	0/8 a
Control	0/6 a	0/8 a

[z] Number of symptomatic plants/total number of inoculated plants. Letters indicate significant difference ($P \leq 0.05$).

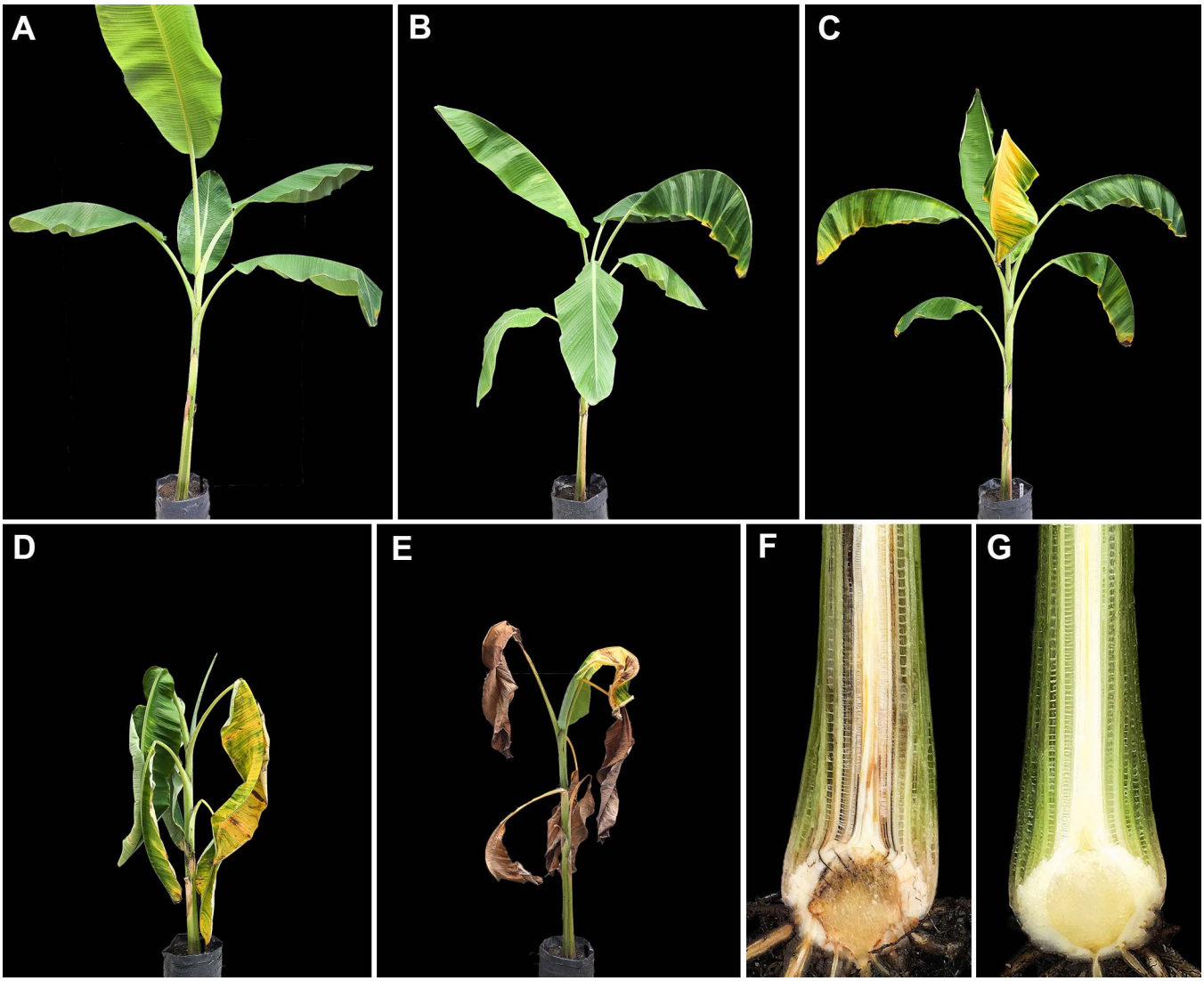

Fig. 3. Symptom progression in potted Kepok banana plants pseudostem inoculated with various concentrations of *Ralstonia syzygii* subsp. *celebesensis*. **A,** Control plant remained healthy. **B,** First symptoms expressed, leaf wilt. **C,** Leaf wilt and chlorosis. **D,** Leaf folding at the petiole, wilt, and chlorosis. **E,** Leaf wilt, chlorosis, and necrosis. **F,** Internal vascular discoloration. **G,** Pseudostem of control plant remained healthy.

DISCUSSION

This study confirms that inoculum concentrations from 10^{10} to 10^{2} CFU/ml of *R. syzygii* subsp. *celebesensis* express Blood disease symptoms in potted Kepok plants after 14 days. Both the male and female parts of a banana inflorescence, the snapped rachis, and deflowered fingers act as infection courts for *R. syzygii* subsp. *celebesensis* in Kepok and Cavendish varieties. When applied to specific banana inflorescence parts, dye directly enters the plant's internal vascular system, supporting the notion that these plant parts contain open xylem vessels.

A concentration as low as 10^{2} CFU/ml of *R. syzygii* subsp. *celebesensis* is able to induce Blood disease in potted Kepok bananas. This inoculum level was lower than previously reported results in which no wilting symptoms developed after inoculation of banana stems with 10^{0} to 10^{3} CFU/ml of *R. syzygii* subsp. *celebesensis* (Baharuddin 1994). No data are available for comparison regarding low bacterial concentrations initiating disease symptoms of bacterial wilts of Moko wilt of banana caused by *R. solanacearum*. Our findings demonstrate that inoculum with a low concentration of viable *R. syzygii* subsp. *celebesensis* cells can reliably initiate Blood disease after stem inoculation.

TABLE 3. Distance of dye movement in centimeters through rachis and true stem xylem measured 24 h after application of dye to various inflorescence plant parts

Treatment	Rep 1	Rep 2	Rep 3	Average
Male flower cushion	42	80	18	47
Male bract scar	9	35	25	23
Female bract scar	160	150	190	167
Snapped rachis	52	190	35	92
Control, female	0	0	N/A[z]	0
Control, male	0	0	N/A	0

[z] N/A = not applicable.

Externally applied dye can directly enter the xylem of banana plants through parts of the inflorescence naturally revealed through abscised flowers and bracts and through wounds that expose the xylem, such as those that occur during deflowering or denaveling. Dye uptake studies are commonly used to show xylem function, and the pattern of observed tissue staining is presumed to replicate the flow of xylem fluid (Bondada et al. 2005; During et al. 1987). Dye applied to male and female bract scars, the male flower cushion, and the snapped rachis moved a significant distance into the plant through the xylem of the rachis and true stem. The pattern of dye staining indicates that the xylem vessels connected to these specific plant parts are open and not just subject to the effect of capillary action but are affected by significant diurnal variation in xylem pressure exhibiting a negative hydrostatic gradient during the day (Kallarackal et al. 1990). This suggests that the open xylem vessels can suck in bacteria and distribute them internally in the plant, where they can initiate infection.

Dye studies demonstrate that the freshly emerged female flowers and the wounds generated through deflowering are directly connected to the xylem within the banana fingers. Dye applied to the tip of the compound tepal and the freshly deflowered finger wound

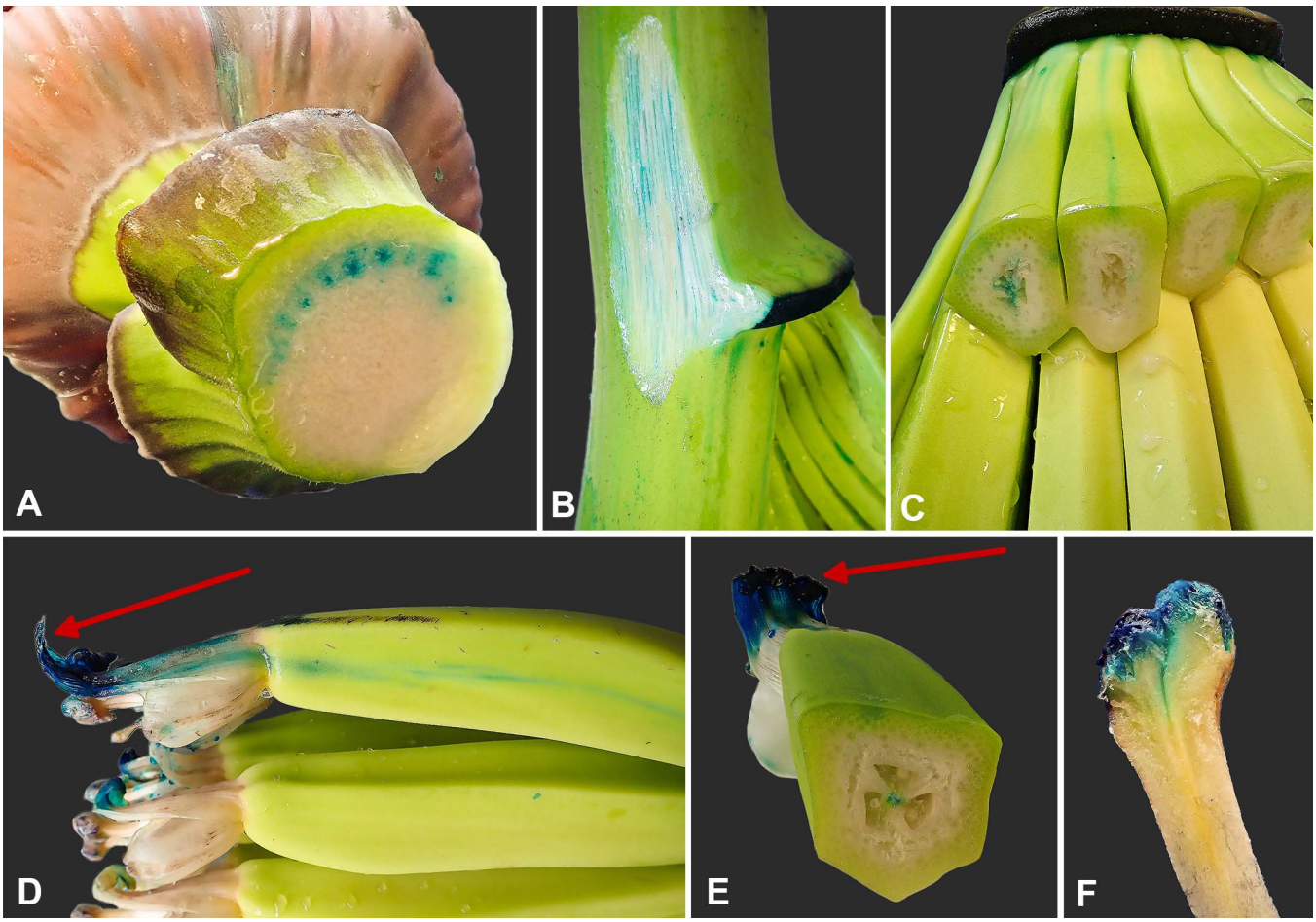

Fig. 4. Uptake of blue dye 24 h after application on various parts of the inflorescence of Cavendish. **A,** Dye applied to the male bell flower cushion was visible inside the rachis. **B and C,** Dye applied to the female bract scars stained the rachis tissue, vascular tissue in the finger skin, and the finger pulp. **D and E,** Dye applied to the compound tepal tip can be seen in the banana finger skin, vascular system, and finger pulp. **F,** Dye applied to the stigma did not enter the plant vascular system.

entered the xylem of the fingers, providing evidence that the xylem of these plant parts is open and subject to a negative hydrostatic pressure gradient. In contrast, the dye did not reach the vascular system through a female flower's stigma and style tube, confirming that the stigma and style receptive to the growth of pollen tubes are not directly connected with xylem (Amah et al. 2021).

The results of the bacterial inoculation studies align with the findings of the dye studies. All plants parts shown during the dye studies to have open xylem vessels subject to a negative pressure gradient when inoculated with *R. syzygii* subsp. *celebesensis* resulted in the banana plant developing Blood disease symptoms. The combined results support the hypothesis that bacteria deposited on the respective plant part may be pulled into the plant's xylem. Once inside the plant's xylem, plant-infecting *Ralstonia* spp. thrive in the flowing xylem fluid of a susceptible host, initiating disease in a systemic manner (Lowe-Power et al. 2018).

Insects have been hypothesized to mechanically transfer *R. syzygii* subsp. *celebesensis* from diseased to healthy male banana bells, thereby spreading the disease (Buddenhagen 2009; Stover and Espinoza 1992). This hypothesis was derived from field-based observations of oozing and necrosis of the male bell and insect behavior associated with development of Blood disease. In contrast, our study provides evidence that both the male and female parts of the banana inflorescence can act as infection courts for Blood disease. Inoculation of Kepok and Cavendish female flowers resulted in the development of banana Blood disease, and the dye studies confirmed a pathway of open xylem at the female flower into the finger xylem. Our findings support previous studies that suggested infection of *R. syzygii* subsp. *celebesensis* through female banana flowers resulted in Blood disease (Gäumann 1921; Hermanto and Emilda 2013).

The question remains as to how the bacterium is transferred to inflorescence infection courts. The hypothesis that insects mechanically transfer the bacterium from diseased to healthy banana inflorescences is supported by studies confirming the presence of *R. syzygii* subsp. *celebesensis* on the bodies of insects from diseased fields and observations that insects frequent both the male and female parts of the inflorescence (Leiwakabessy 2003; Mairawita et al. 2015; Tinzaara et al. 2006). Our results show that bacteria deposited on an infection court are sucked into the plant xylem, where they can establish an infection resulting in disease. This finding implies that transmission to these plant parts is purely mechanical and nonspecific and that transmission of *R. syzygii* subsp. *celebesensis* can inadvertently occur through mechanical transfer by rain splash and any insect, bird, bat, or human through contact with inoculum from a contagious inflorescence.

The present investigation demonstrated that the management practices of denaveling and deflowering creates infection courts. The freshly snapped rachis drips sap and xylem fluid, as do the freshly deflowered fingers, although to a lesser extent. Insects tend to be abundant in banana fields, flying randomly from plant to plant, and they may find these fluids attractive. Deflowering normally conducted by hand in a Cavendish plantation would also carry the risk of mechanically transferring the bacteria from an infected inflorescence to a healthy one.

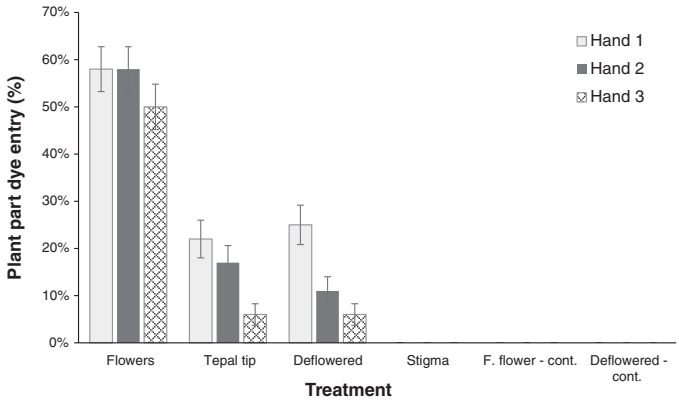

Fig. 5. Externally applied dye entered various female inflorescence plant parts of the three most recently revealed hands, hand one being closest to the male bell and the most recently revealed. Bars = standard error.

This study has established that infection of *R. syzygii* subsp. *celebesensis* can occur through the female and male parts of the banana inflorescence and the recently snapped rachis. Plant physiology leads us to the conclusion that the male bell plays a critical role in disease transmission. The length of time the male bell remains susceptible to infection and attractive to insects far exceeds that of the female inflorescence or snapped rachis (Nakato et al. 2014). After emergence, female banana flowers and bract scars are successively revealed over approximately 2 weeks and they readily dry and become unattractive to insects, as does the snapped rachis (Nakato et al. 2014; Stover and Simmonds 1987). In contrast, Cavendish and Kepok banana male bells reveal one to two fresh bract scars and rows of flower cushions daily, which act as infection courts over a period of approximately 3 months (Stover and Simmonds 1987). This extended length of time that the male bell remains attractive to insects and vulnerable to infection supports the hypothesis that the male bell is the primary infection court for insect-mediated transmission of *R. syzygii* subsp. *celebesensis*.

Blood disease continues to cause crop losses across Indonesia and Malaysia and is an emerging threat to banana production in Southeast Asia. Blood disease certainly poses a significant risk to the cultivation of commercially grown Cavendish and Kepok bananas and non-commercial backyard-grown banana varieties in locations where the disease occurs. This study provides insights into the susceptibility of various parts of the inflorescence to infection by *R. syzygii* subsp. *celebesensis*. Prevention or reduction of insect visitation to these infection courts may reduce transmission of the bacterium. Therefore, using sealed cloth bunch bags at emergence to protect the female inflorescence and promptly removing the male bell using a forked stick so as not to contaminate the cut surface may reduce disease incidence in the field. However, this needs extensive field testing before being recommended as a management practice.

Our study has filled some of the knowledge gaps concerning the infection biology of Blood disease, although further work is required to gain a complete picture of the disease cycle. It is critical to understand infection and transmission pathways, including the role of tool transmission, the role of contaminated planting materials in long-distance dispersal, and the role of root infection via contaminated soil, water, or root-to-root contact. This knowledge will enable the development of a more comprehensive set of tools and management practices to improve disease management in areas in which the disease occurs and help to define quarantine strategies for areas still free of banana Blood disease.

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